

# *In vitro* and *in vivo* effects of beneficial vaginal lactobacilli on pathogens responsible for urogenital tract infections

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The aim of this work was to evaluate the effects of beneficial human vaginal lactobacilli (Lb) on urogenital pathogens through *in vitro* and *in vivo* experiments. Co-aggregative and antimicrobial properties between five vaginal Lb strains and urogenital pathogens or potential pathogens (*Streptococcus agalactiae*, *Staphylococcus aureus* and *Candida albicans* strains) were assayed. Also, associative cultures of Lb strains and *S. agalactiae* were performed and bacterial growth, pH, lactic acid and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) were determined at different times. Based on the results obtained, the *in vivo* studies were assayed in mice with *Lactobacillus gasseri* CRL 1509 or *Lactobacillus salivarius* CRL 1328 inoculated intravaginally (i.v.) and then challenged i.v. with *S. agalactiae*. Results were analysed by ANOVA (repeated measures and general linear models). Most of the Lb strains increased the percentage of aggregation of *S. agalactiae* strains. Only one strain (*Lactobacillus reuteri* CRL 1324) positively affected the aggregation of *S. aureus* and none increased the aggregation of *C. albicans*. The inhibition of the growth of *S. agalactiae* strains by production of organic acids by lactobacilli was evidenced. The Lb–*S. agalactiae* co-cultures showed a significant inhibition of the pathogen after 4 h and 8 h of incubation. Parallel increases in lactic acid and H<sub>2</sub>O<sub>2</sub> levels were observed. However, in the experimental murine model, no significant differences were obtained in the number of streptococci recovered from the vaginal tract of control mice and those inoculated with Lb. In conclusion, vaginal Lb exhibited *in vitro* co-aggregative and antimicrobial effects on *S. agalactiae* strains, suggesting that they could be promising candidates for protection against *S. agalactiae* challenge. However, as these effects were not evidenced in the murine model used, further animal studies under different experimental conditions should be conducted to evaluate the preventive effect of Lb against challenge with *S. agalactiae*.

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## INTRODUCTION

The vaginal microbiome of healthy women of reproductive age is constituted by communities with a low microbial diversity within each individual and between different subjects at the genus level (Ravel *et al.*, 2011). Studies of the human microbiome have revealed that different *Lactobacillus* species are predominant but mutually exclusive in the vagina (Human Microbiome Project Consortium, 2012). Vaginal lactobacilli play a significant role in maintaining the balance of the dynamic ecosystem of the urogenital tract.

**Abbreviations:** i.v., intravaginal(ly); LAB, lactic acid bacteria; UGTI, urogenital tract infection.

One supplementary table and two supplementary figures are available with the online version of this paper.

Lactobacilli can protect against urogenital tract infections (UGTI) by preventing pathogenic or potentially pathogenic micro-organisms adhering to the vaginal epithelial surface, forming biofilm, co-aggregating with pathogens, producing antimicrobial substances [organic acids, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and bacteriocins], competing for nutrients and stimulating the local immune response (Ocaña *et al.*, 1999a; Juárez Tomás *et al.*, 2003; Martín *et al.*, 2008; Nader-Macías *et al.*, 2010). Co-aggregation between lactobacilli and urogenital pathogens is a process that can prevent pathogen entry and colonization of the mucosal sites of the host (Boris *et al.*, 1997, 1998; Schachtsiek *et al.*, 2004; Schellenberg *et al.*, 2006). Also, co-aggregation could result in prolonged exposure of pathogens to antagonistic compounds produced by lactobacilli, which could reduce pathogen viability (Saunders *et al.*, 2007; Jones & Versalovic, 2009; McMillan *et al.*, 2011).

An imbalance of the urogenital tract microbiota can produce a decrease in *Lactobacillus* population and a significant increase in pathogens or potential pathogens, leading to a higher susceptibility to UGTI (Ravel *et al.*, 2011). The following agents are involved in UGTI: aerobic bacteria associated with aerobic vaginitis (e.g. *Streptococcus agalactiae*, *Staphylococcus aureus*, *Escherichia coli* and enterococci), anaerobic and facultative bacteria related to bacterial vaginosis (e.g. *Gardnerella vaginalis*, *Atopobium vaginae*, *Prevotella bivia*, *Mobiluncus* spp. and *Mycoplasma* spp., among others), yeasts associated with vulvovaginitis (e.g. *Candida* spp.), and several bacteria, viruses and protozoa responsible for sexually transmitted infections (Schwebke & Burgess, 2004; Fredricks *et al.*, 2005; Larsson & Forsum, 2005; Zhou *et al.*, 2009; Donders *et al.*, 2009; Laufer *et al.*, 2010).

UGTI in women generate high morbidity and recurrence rates, probably due to failure of the conventional antimicrobial treatments to restore the *Lactobacillus*-dominated vaginal microbiome. For this reason, the intravaginal (i.v.) administration of pharmabiotic products containing beneficial lactobacilli could be a promising alternative strategy for the prevention or eradication of UGTI (Barrons & Tassone, 2008; Martín *et al.*, 2008). Pharmabiotics are defined as 'live or dead microorganisms as well as microbial constituents and metabolites which can beneficially interact with the host' (Shanahan *et al.*, 2009). Our research group has isolated and selected autochthonous lactobacilli from human vagina that could be included as active pharmaceutical ingredients in pharmabiotic products to re-establish the balance of the urogenital tract (Ocaña *et al.*, 1999a, b; Juárez Tomás *et al.*, 2003, 2005, 2011).

Both *in vitro* studies and animal models are required to assess the beneficial characteristics of the different strains (Reid *et al.*, 2003; Yao *et al.*, 2007; Saksena *et al.*, 2011; De Gregorio *et al.*, 2012). In the present work, the *in vitro* co-aggregative and antimicrobial properties of five different vaginal *Lactobacillus* strains, previously selected for their beneficial properties, were evaluated on some pathogens responsible for UGTI. Furthermore, the preventive effect of vaginal *Lactobacillus* colonization against i.v. challenge with *S. agalactiae* was studied in a murine model.

## METHODS

**Micro-organisms and culture conditions.** Five *Lactobacillus* strains, originally isolated from human vagina in Tucumán, Argentina (Ocaña *et al.*, 1999a), were previously selected on the basis of their beneficial properties. *Lactobacillus gasseri* CRL (Centro de Referencia para Lactobacilos Culture Collection) 1263, *L. gasseri* CRL 1509, *Lactobacillus reuteri* CRL 1324, and *Lactobacillus rhamnosus* CRL 1332 produce H<sub>2</sub>O<sub>2</sub> (Juárez Tomás *et al.*, 2011) while *Lactobacillus salivarius* CRL 1328 produces salivaricin CRL 1328 (a bacteriocin) (Ocaña *et al.*, 1999b; Vera Pingitore *et al.*, 2009). Furthermore, *L. reuteri* CRL 1324 and *L. rhamnosus* CRL 1332 are biofilm-forming strains (Terraf *et al.*, 2012).

Urogenital pathogens (*Streptococcus agalactiae* NH 16, NH 17, NH 18 and GB 96 strains, *Staphylococcus aureus* and *Candida albicans* F 1 and F 78 strains) were isolated from patients with UGTI at the Nuevo

Hospital 'El Milagro' (Salta, Argentina) and at the Instituto de MicroBiología 'Luis Verna' of the Universidad Nacional de Tucumán.

Micro-organisms were stored in milk-yeast extract [% (w/v): 13 non-fat milk, 0.5 yeast extract and 1 glucose] at -20 °C. Before experimental use, each *Lactobacillus* strain was grown in LAPTg (yeast extract/peptone/tryptone/Tween 80/glucose) broth (Raibaud *et al.*, 1973) at 37 °C for 24 h and subcultured twice in the same medium at 37 °C for 12 h. The pathogens were cultured in LAPTg at 37 °C for 12 h and then all the strains, with the exception of *C. albicans* F 1, were subcultured at 37 °C in the same medium for 8 h, a time in which they reached the late exponential phase. *C. albicans* F 1 was subcultured for 12 h. All cultures were performed under static conditions.

### Co-aggregation between lactobacilli and urogenital pathogens.

Co-aggregation between lactobacilli and pathogens was studied as previously described (Ocaña & Nader-Macías, 2002). Briefly, cell suspensions of lactobacilli and pathogens in sterile PBS (g l<sup>-1</sup>: 8.0 NaCl; 0.34 KH<sub>2</sub>PO<sub>4</sub>; and 1.21 K<sub>2</sub>HPO<sub>4</sub>; pH 6.5) were adjusted to an optical density at 600 nm (OD) of 0.6 ± 0.02 (Spectronic 20; Bausch and Lomb) and combined (1:1). Co-aggregation was spectrophotometrically monitored every 30 min for 4 h. Controls of suspensions of each individual micro-organism were employed. Aggregation percentage was calculated as 1 - (OD<sub>final</sub>/OD<sub>initial</sub>) × 100, where OD<sub>initial</sub> is the OD at time t=0, and OD<sub>final</sub> is the OD at time t (in minutes) after the beginning of the assay.

### *In vitro* antagonistic effects of lactobacilli and urogenital pathogens

**Agar plate diffusion method.** The antimicrobial effect of bacterial supernatants was evaluated with the agar plate diffusion technique (Juárez Tomás *et al.*, 2004, 2011), using lactobacilli and pathogens as indicators and/or as producers of antimicrobial substances. Briefly, microbial supernatants were left untreated, neutralized with 2 N NaOH, or neutralized and treated with catalase (1000 U ml<sup>-1</sup>). Then, untreated and treated supernatants were placed in 4 mm diameter holes in agar plates containing the different indicator micro-organisms (10<sup>7</sup>-10<sup>8</sup> c.f.u. ml<sup>-1</sup>). The subsequent steps of the diffusion technique were performed as previously described (Juárez Tomás *et al.*, 2011).

### Associated cultures of vaginal lactobacilli and *S. agalactiae*

**NH 17.** Co-cultures of lactobacilli (*L. gasseri* CRL 1263, *L. reuteri* CRL 1324, *L. salivarius* CRL 1328 or *L. gasseri* CRL 1509) and *S. agalactiae* NH 17 were performed in LAPTg broth at 37 °C under static conditions. Two inoculum concentrations of lactobacilli (around 10<sup>6</sup> and 10<sup>8</sup> c.f.u. ml<sup>-1</sup>) and one of *Streptococcus agalactiae* (10<sup>5</sup> c.f.u. ml<sup>-1</sup>) were assayed. Pure cultures (employed as control cultures) were grown under the same culture conditions. At different times during the cultures, bacterial growth, pH (as an indirect measure of acid production) and levels of antimicrobial metabolites (H<sub>2</sub>O<sub>2</sub> and organic acids) were assayed.

Bacterial growth was determined by the standard plate dilution method, using the selective culture medium De Man-Rogosa-Sharp (MRS) agar (Merck) (De Man *et al.*, 1960) at pH 5.5 for lactobacilli and Bacto Todd-Hewitt (TH) agar at pH 7.8 (Becton Dickinson) for streptococci. The plates were incubated at 37 °C for 48 h and the number of c.f.u. ml<sup>-1</sup> was determined.

The levels of organic acids in the supernatants of pure and associated cultures were quantified by HPLC as previously described (Juárez Tomás *et al.*, 2011). H<sub>2</sub>O<sub>2</sub> production during the growth was evaluated by a chromogenic method on plates of MRS agar medium supplemented with 1 mM tetramethylbenzidine (TMB) and 2 U ml<sup>-1</sup> horseradish peroxidase (MRS-TMB) (Juárez Tomás *et al.*, 2004).

Briefly, micro-organisms were inoculated on the surface of MRS–TMB plates, which were then incubated under microaerophilic conditions at 37 °C for 48 h. Colonies of H<sub>2</sub>O<sub>2</sub>-producing strains turned blue after exposure to air for 30 min and the intensity of the blue colour allowed the classification of H<sub>2</sub>O<sub>2</sub> production by the strains as (+ +) strong, (+) moderate, (+ w) weak and (–) negative.

#### **In vivo effects of lactobacilli on *S. agalactiae* NH 17.**

An experimental murine model was employed to evaluate the preventive effect of the previous i.v. inoculation of *L. salivarius* CRL 1328 and *L. gasseri* CRL 1509 on the i.v. challenge with *S. agalactiae* NH 17.

**Animals.** Two-month-old female BALB/c mice, weighing 25–30 g, from the inbred colony of CERELA (Centro de Referencia para Lactobacilos) were used. Animals were housed in plastic cages and fed *ad libitum* with a conventional balanced diet, keeping their environmental conditions constant. To induce a pseudo-oestrous condition and promote bacterial colonization, all mice received a weekly subcutaneous injection of 0.02 mg  $\beta$ -oestradiol 17-valerate (Sigma) dissolved in 100  $\mu$ l sesame oil (Sigma) (Furr *et al.*, 1989; Cheng *et al.*, 2005; Rahman *et al.*, 2007) throughout the experiment. The experimental protocol was independently repeated three times using at least three animals for each experimental group at each sampling time. The Institutional Laboratory Animal Care and Use Committee of CERELA approved the experimental CRL-BIOT-LMP-2011/2A protocol used in this work.

**Vaginal inoculation procedure.** The animals were randomly assigned to five experimental groups: (a) lactobacilli (Lb)–pathogen (Pt)-treated mice (inoculated with *L. salivarius* CRL 1328 or *L. gasseri* CRL 1509 and later challenged with *S. agalactiae* NH 17), (b) Pt-treated mice (inoculated with saline and later challenged with *S. agalactiae* NH 17) and (c) Lb-treated mice (inoculated with *L. salivarius* CRL 1328 or *L. gasseri* CRL 1509 and later with saline) (Fig. 1).

Forty-eight hours after oestradiol administration, Lb–Pt-treated mice and Lb-treated mice were i.v. inoculated with 10<sup>7</sup>–10<sup>8</sup> c.f.u. *L. salivarius* CRL 1328 or *L. gasseri* CRL 1509 twice a day (with 10 h in between) for 2 days, while Pt-treated mice were inoculated with saline [0.85 % (w/v) NaCl] on these days. The *Lactobacillus* inoculum was prepared from the cell pellet of the third subculture (performed in LAPTg broth for 12 h at 37 °C as described above), resuspended in 50  $\mu$ l agarized peptone [% (w/v): 1 meat peptone, 1.5 agar; Britania Laboratories], as previously described (De Gregorio *et al.*, 2012).

Fourteen hours after the last *Lactobacillus* inoculation, Lb–Pt-treated mice and Pt-treated mice were i.v. challenged with a single dose of around 5  $\times$  10<sup>5</sup> c.f.u. *S. agalactiae* NH 17 (in 20  $\mu$ l saline), while Lb-treated mice were inoculated with 20  $\mu$ l saline. Before challenge, *S. agalactiae* NH 17 was cultured overnight on sheep blood agar plates and then subcultured in TH broth for 9 h at 37 °C. The *Streptococcus* inoculum was prepared with bacterial pellets from the second subculture, which were washed and resuspended in saline. Fig. 1 shows the experimental groups, the sequence of vaginal inoculation and the sampling days of the experimental design. Lactobacilli inoculation and pathogen challenge are indicated by two different time lines.

**Sampling and analytical procedures.** Every sampling day, vaginal washings were obtained under sterile conditions, using automatic pipettes with tips loaded with 50  $\mu$ l saline. Seven vaginal washes with saline were pooled from each mouse to be later used for the different determinations. Subsequently, mice were killed by cervical dislocation and dissected to aseptically remove the vaginal tissue.

For cytological studies, 20  $\mu$ l aliquots of vaginal washes were spread onto glass slides and stained with the May–Grünwald–Giemsa

technique. Then, the vaginal smears and the histological slides (described below) were evaluated by light microscopy (at  $\times$  400) to determine the oestrous state of mice (Silva de Ruiz *et al.*, 2001) and the production of adverse effects.

Histological studies of vaginal tissue were performed according to standard methods with haematoxylin–eosin stain (Biopur) (Silva de Ruiz *et al.*, 2003). Pictures were taken with an Axio Scope A1 Carl Zeiss microscope. The images were processed using Axio-Vision release 4.8 software.

Bacterial counts in vaginal washes were determined by the serial dilution method by plating in selective media: MRS (pH 5.5) supplemented with 1  $\mu$ g ml<sup>–1</sup> tetracycline or 10  $\mu$ g ml<sup>–1</sup> vancomycin (Sigma), to quantify *L. gasseri* CRL 1509 or *L. salivarius* CRL 1328, respectively (De Gregorio *et al.*, 2012), and chromID Strepto B agar (bioMérieux) to quantify *S. agalactiae* NH 17 (Poisson *et al.*, 2011). The plates were incubated under aerobic conditions at 37 °C for 48 h.

**Statistical analysis.** For the co-aggregation and associative cultures assays, ANOVA using a repeated measures model was applied to determine the main and interaction effects of factors (strain and time). Significant differences between the kinetics of co-aggregation or between the growth curves were determined by Fisher's least significant difference (LSD) test, using the InfoStat vs 2012 software. A *P*-value <0.05 was considered as statistically significant. For *in vivo* studies, ANOVA using a general linear model was applied to determine the main and interaction effects of factors (experimental group and day post-inoculation). Significant differences (*P*-value <0.05) between mean values were determined by Tukey's test, using MINITAB statistical software (version 15 for Windows).

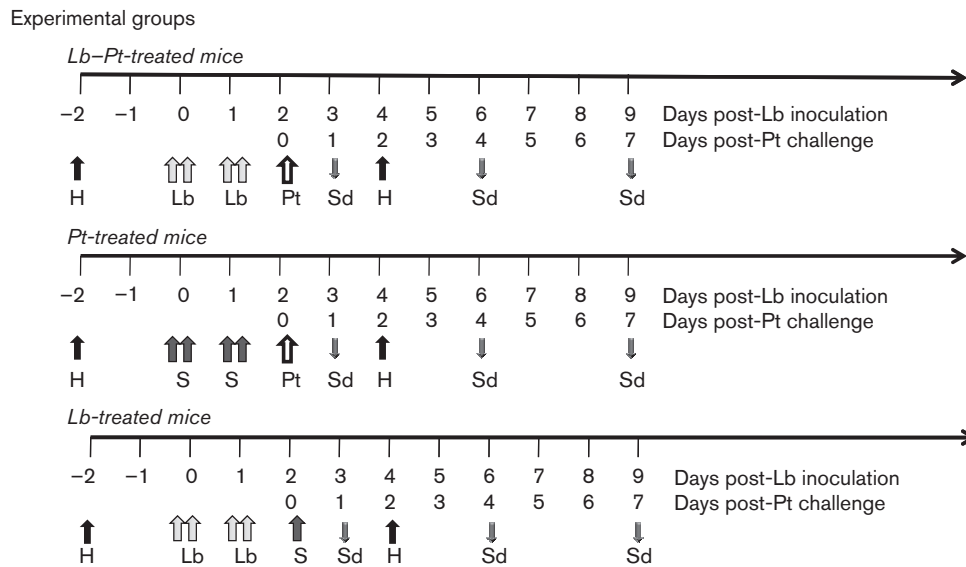
## RESULTS

### Co-aggregation between lactobacilli and pathogens

The degree of aggregation of all the pathogens was significantly affected by the *Lactobacillus* strain (*P*<0.01) and by the length of the aggregation assay (*P*<0.0001) (Fig. 2). *Lactobacillus*–pathogen co-aggregation was considered significant when the co-aggregation percentage was significantly higher (*P*<0.05) than the self-aggregation percentage of each pathogen strain.

Out of the five vaginal *Lactobacillus* strains studied, only *L. gasseri* CRL 1509, *L. gasseri* CRL 1263 and *L. reuteri* 1324 were able to co-aggregate significantly (*P*<0.05) with at least two of the four *S. agalactiae* strains evaluated (Fig. 2A). Maximal co-aggregation percentage between *L. salivarius* CRL 1328 and *S. agalactiae* strains was slightly higher than *S. agalactiae* self-aggregation percentages (Fig. 2A).

The aggregation percentage of *S. aureus* was significantly higher (*P*<0.05) only in the presence of *L. reuteri* CRL 1324 (Fig. 2B). On the other hand, the self-aggregation percentages of the two *C. albicans* strains studied were significantly higher (*P*<0.05) than the percentages of co-aggregation between the yeasts and all *Lactobacillus* strains evaluated, except for *C. albicans* F 78 in the presence of *L. gasseri* CRL 1509 (Fig. 2C).



**Fig. 1.** Experimental groups, sequence of inoculation and sampling days of the mice protocol applied. H, Hormone administration (0.02 mg  $\beta$ -oestradiol 17-valerate). Each arrow indicates one inoculation of lactobacilli in 50  $\mu$ l containing  $10^7$ – $10^8$  c.f.u. (Lb, *L. gasseri* CRL 1509 or *L. salivarius* CRL 1328), pathogen (20  $\mu$ l containing around  $5 \times 10^5$  c.f.u.; Pt, *S. agalactiae* NH 17) or saline (S) to mice in the following experimental groups: Lb–Pt-treated mice, Pt-treated mice and Lb-treated mice. Sd, Sampling day.

All the pathogenic strains tested, with the exception of *S. agalactiae* NH 16 and NH 18, showed lower aggregation percentages in the presence of *L. rhamnosus* CRL 1332 than their self-aggregation percentages (Fig. 2A–C). In general, vaginal *Lactobacillus* strains evidenced higher self-aggregation abilities than pathogenic micro-organisms (Fig. 2D), except in the case of *Candida* strains, which were the micro-organisms with highest self-aggregation values.

### **In vitro antagonistic effects of lactobacilli and urogenital pathogens**

In the agar plate diffusion method, only *L. gasseri* CRL 1509 inhibited the growth of *S. agalactiae* NH 17 and GB 96, while *L. reuteri* CRL 1324 inhibited *S. agalactiae* GB 96 (data not shown). *L. gasseri* CRL 1509 produced the highest well-defined inhibition halo (8 mm diameter) on the growth of *S. agalactiae* NH 17. The inhibitory activity of the two *Lactobacillus* strains disappeared after the neutralization of the supernatants, which indicates that organic acids are responsible for the antagonistic effect. Moreover, when the inhibitory effect of the pathogens was assayed on *Lactobacillus* strains, *L. reuteri* CRL 1324 was the only strain inhibited by a pathogenic micro-organism (*S. agalactiae* NH 16).

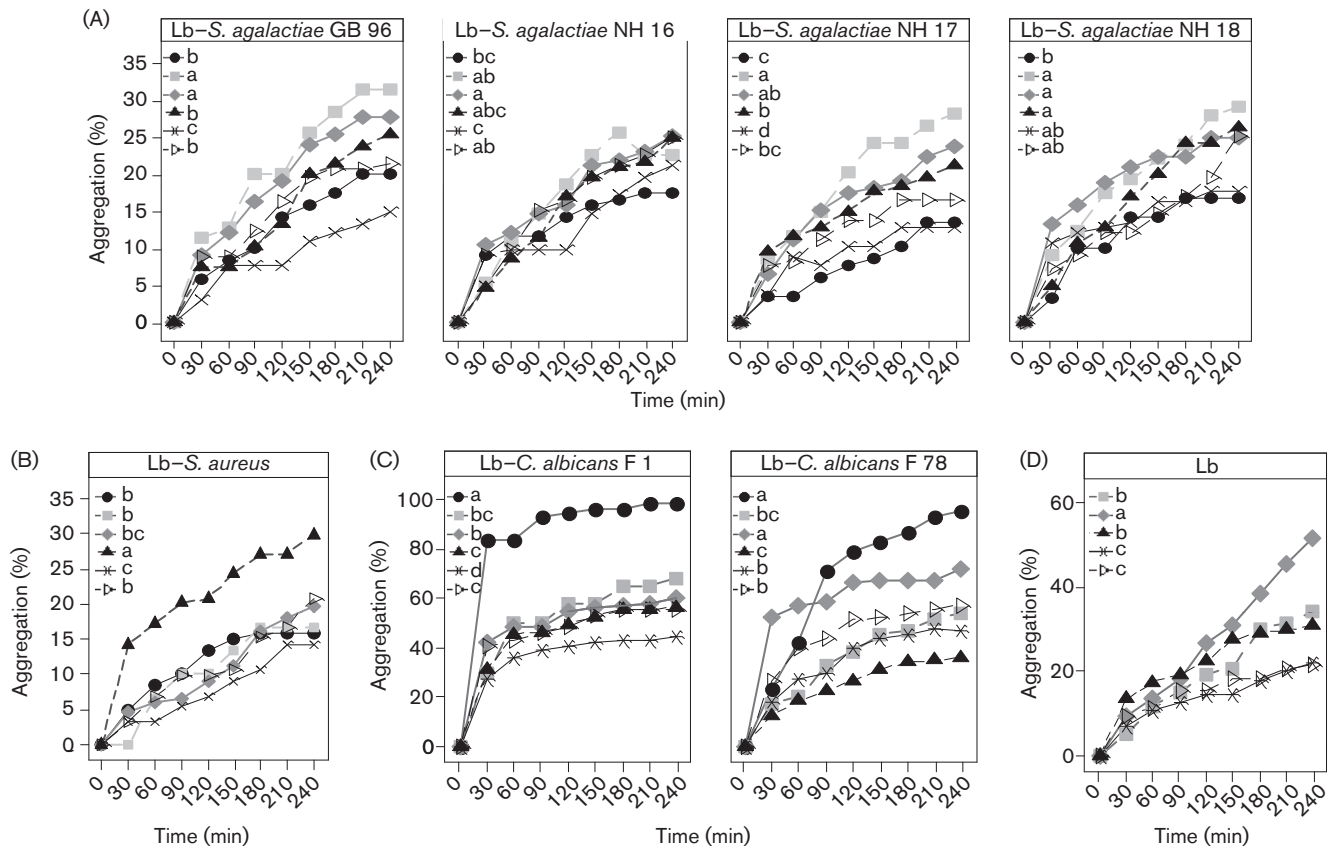
### **Associated cultures of lactobacilli and *S. agalactiae* NH 17**

On the basis of the results obtained in the studies described above, co-cultures of *L. gasseri* CRL 1263, *L. reuteri* CRL

1324, *L. salivarius* CRL 1328 or *L. gasseri* CRL 1509 and *S. agalactiae* NH 17 were carried out to further evaluate the degree of interaction.

The lactobacilli and *S. agalactiae* NH 17 co-cultures showed a significant inhibition ( $P < 0.05$ ) of pathogen growth at 4 h and 8 h of incubation with the high and low *Lactobacillus* inocula, respectively. The lowest numbers of pathogen cells (reduction of 4–5 log units) were observed after 12 h of co-culture with *L. gasseri* CRL 1263, *L. salivarius* CRL 1328 and *L. gasseri* CRL 1509 at both inoculum concentrations. *L. reuteri* CRL 1324 inhibited *S. agalactiae* NH 17 (4 log units) after 24 h only with the high inoculum. *L. gasseri* CRL 1509 and *L. salivarius* CRL 1328 were the strains that showed the strongest inhibitory effect against *S. agalactiae* NH 17. On the other hand, the number of viable lactobacilli in mixed and pure cultures was similar (Fig. 3).

A marked decrease in pH in both pure and mixed cultures of lactobacilli and *S. agalactiae* was observed up to 8 h of incubation, followed by a slight decrease up to 48 h. Parallel increases in lactic acid levels were observed. *L. gasseri* CRL 1509 and *L. gasseri* CRL 1263 [both homofermentative lactic acid bacteria (LAB)] showed the highest lactic acid concentrations after 12 h ( $10.55 \pm 0.18$  g l<sup>-1</sup>) and 24 h ( $8.83 \pm 0.41$  g l<sup>-1</sup>) of incubation, respectively. On the other hand, *L. reuteri* CRL 1324 (a heterofermentative LAB) and *L. salivarius* CRL 1328 (a homofermentative LAB) produced lower amounts of lactic acid, reaching maximum values after 12 h ( $4.05 \pm 0.28$  g l<sup>-1</sup>) and 48 h ( $5.50 \pm 0.16$  g l<sup>-1</sup>) of incubation, respectively (Fig. 4).



**Fig. 2.** Co-aggregation abilities of *Lactobacillus* strains (Lb) with *S. agalactiae* GB 96, NH 16, NH 17 and NH 18 (A), *S. aureus* (B) and *C. albicans* F 1 and F 78 (C), and self-aggregation of lactobacilli (D) monitored spectrophotometrically and expressed as aggregation percentage. Self-aggregation of pathogen strains (●). Lb–pathogen co-aggregation (A–C) or Lb self-aggregation (D): *L. gasseri* CRL 1263 (○), *L. reuteri* CRL 1324 (▲), *L. rhamnosus* CRL 1332 (×), *L. salivarius* CRL 1328 (▷) and *L. gasseri* CRL 1509 (◆). The data are plotted as the mean values of aggregation percentages. Statistically significant differences ( $P < 0.05$ ) in the kinetics of aggregation of each combination studied are indicated by different letters on the left of each plot. Different scales on the y-axis were used for each group of micro-organisms in order to distinguish the differences between the aggregation kinetics.

However, *L. reuteri* CRL 1324 produced other antimicrobial metabolites, such as ethanol ( $2.46 \pm 0.13$  g l<sup>-1</sup> at 12 h) and acetic acid ( $0.30 \pm 0.034$  g l<sup>-1</sup> at 48 h). *S. agalactiae* NH 17, which belongs to the LAB group, produced lower lactic acid levels than all the *Lactobacillus* strains evaluated ( $3.19 \pm 0.041$  g l<sup>-1</sup> at 12 h). *L. gasseri* CRL 1509 reached the lowest pH values at 8 h of incubation and produced the highest lactic acid concentrations, which were consistent with the marked inhibitory effect on *S. agalactiae* (Figs 3 and 4).

*L. reuteri* CRL 1324 and *L. gasseri* CRL 1263 evidenced strong production of H<sub>2</sub>O<sub>2</sub> in both pure and associated cultures. Weak to moderate H<sub>2</sub>O<sub>2</sub> production was observed in cultures of *L. gasseri* CRL 1509 and *S. agalactiae* NH 17. In contrast, *L. salivarius* CRL 1328 did not produce H<sub>2</sub>O<sub>2</sub> (Table S1, available in the online Supplementary Material). *L. reuteri* CRL 1324, the strain that showed the highest H<sub>2</sub>O<sub>2</sub> production, inhibited *S. agalactiae* at lower levels,

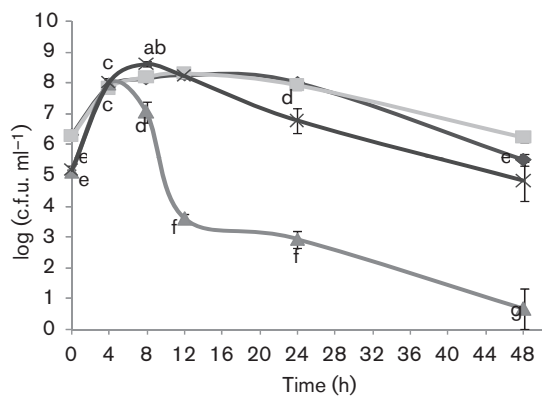
suggesting that lactic acid could be the main antagonistic metabolite responsible for the inhibition of the pathogen (Fig. 4, Table S1).

### **In vivo effects of lactobacilli on *S. agalactiae* NH 17**

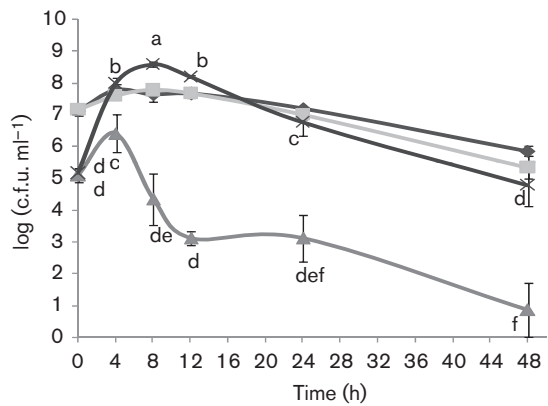
*L. salivarius* CRL 1328 and *L. gasseri* CRL 1509 were selected to evaluate their effect on the vaginal tract against the challenge with *S. agalactiae* in an experimental murine model. The two *Lactobacillus* strains were selected on the basis of the results obtained in the *in vitro* studies.

Seventy-five per cent of Lb–Pt-treated mice and Pt-treated mice were colonized with *S. agalactiae* NH 17. *S. agalactiae* NH 17 was recovered up to the seventh day post-pathogen challenge from vaginal samples of Lb–Pt-treated mice and Pt-treated mice. There were no significant differences ( $P > 0.05$ ) in the number of viable streptococci between

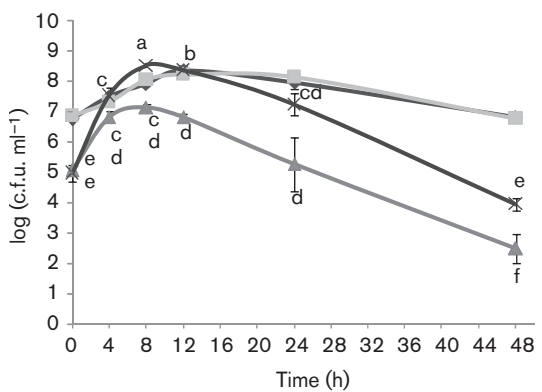
*L. gasseri* CRL 1263 (low inoculum)-*S. agalactiae* NH 17



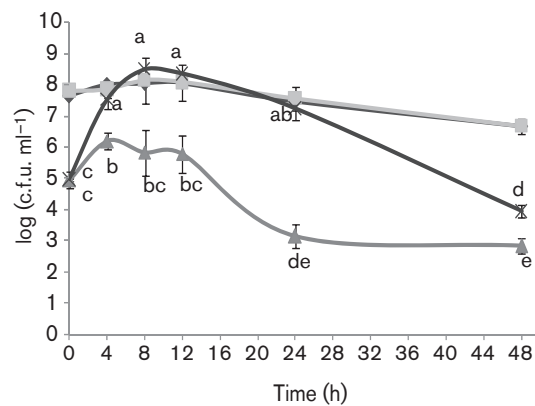
*L. gasseri* CRL 1263 (high inoculum)-*S. agalactiae* NH 17



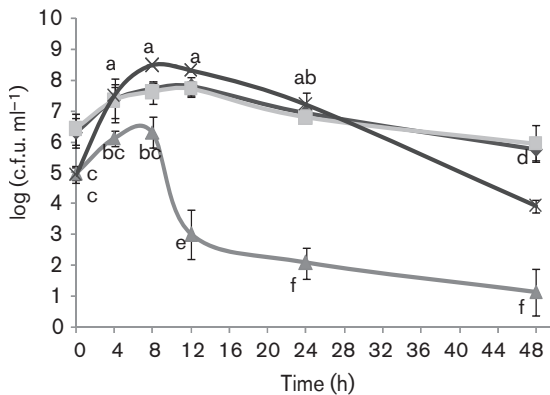
*L. reuteri* CRL1324 (low inoculum)-*S. agalactiae* NH 17



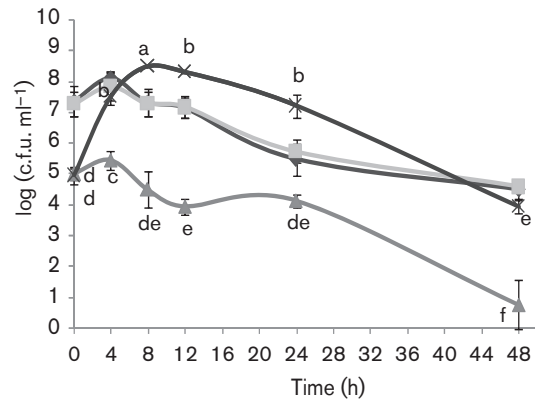
*L. reuteri* CRL1324 (high inoculum)-*S. agalactiae* NH 17



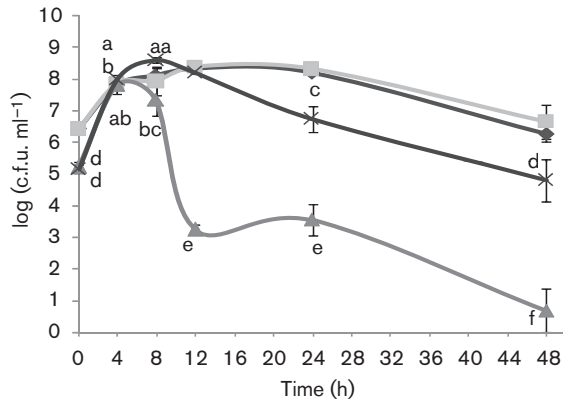
*L. salivarius* CRL 1328 low inoculum-*S. agalactiae* NH 17



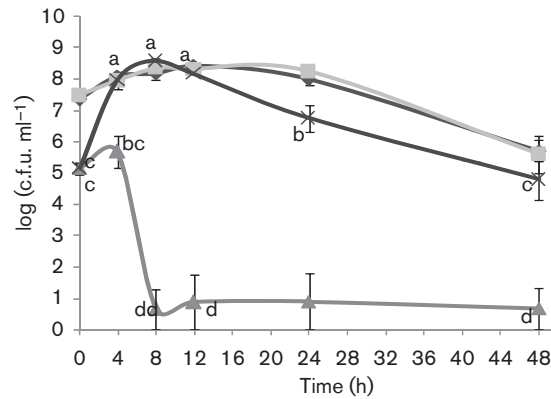
*L. salivarius* CRL 1328 high inoculum-*S. agalactiae* NH 17



*L. gasseri* CRL 1509 (low inoculum)-*S. agalactiae* NH 17



*L. gasseri* CRL 1509 (high inoculum)-*S. agalactiae* NH 17

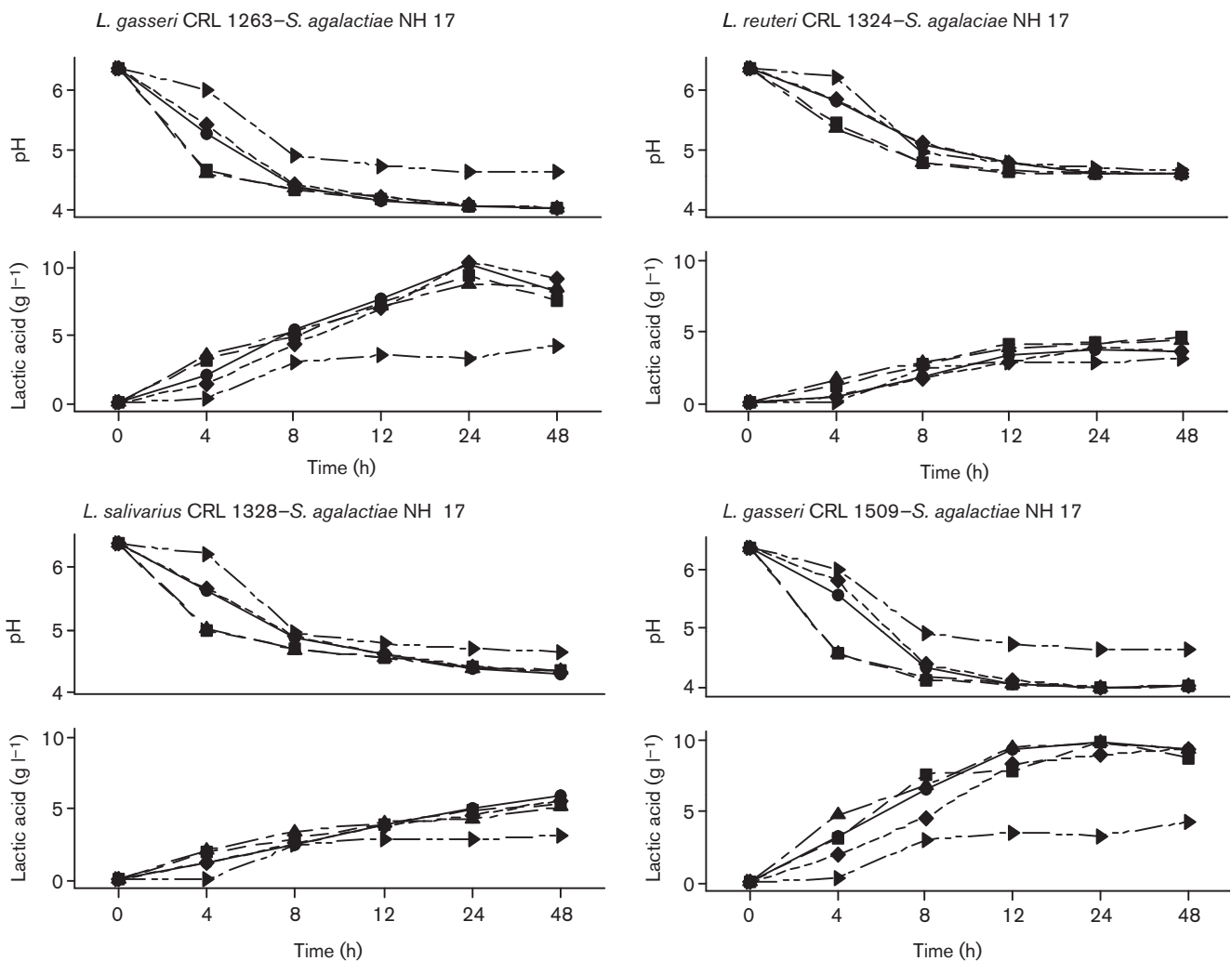


**Fig. 3.** Pure and associative cultures of lactobacilli (*L. gasseri* CRL 1263, *L. reuteri* CRL 1324, *L. salivarius* CRL 1328 or *L. gasseri* CRL 1509) and *S. agalactiae* NH 17. *S. agalactiae* NH 17 in pure (×) and mixed cultures (▲); *Lactobacillus* strains in pure (◆) and mixed cultures (■). Data are plotted as the mean values of viable cell numbers ( $\log \text{c.f.u. ml}^{-1} \pm \text{SEM}$ ). Statistically significant differences between viable cell numbers of *S. agalactiae* NH 17 in pure and mixed cultures at different sampling times are indicated by different letters ( $P < 0.05$ ).

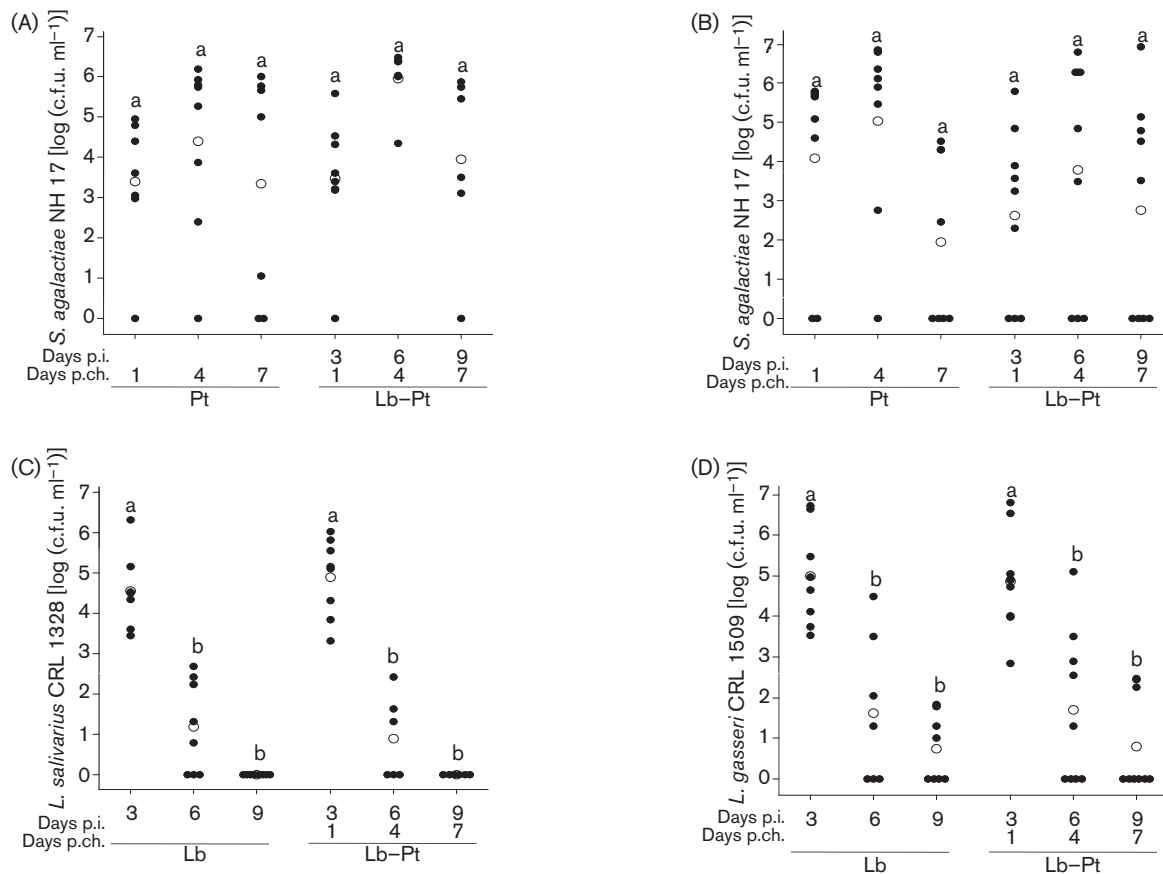
both experimental groups throughout the various sampling days (Fig. 5A, B).

In the vaginal washings of all the Lb–Pt-treated mice and Lb-treated mice, high numbers of viable *L. salivarius* CRL 1328 ( $4.73 \pm 0.24 \log \text{c.f.u. ml}^{-1}$ ) or *L. gasseri* CRL 1509 ( $4.98 \pm 0.28 \log \text{c.f.u. ml}^{-1}$ ) were recovered at 3 days post-inoculation of lactobacilli. In both experimental

groups, a significant decrease ( $P < 0.001$ ) in the number of viable lactobacilli was observed throughout the sampling period. Only *L. gasseri* CRL 1509 persisted in the vaginal tract up to the ninth day after *Lactobacillus* inoculation. No significant differences ( $P > 0.05$ ) were observed in the numbers of vaginal lactobacilli between Lb–Pt-treated mice and Lb-treated mice on different sampling days (Fig. 5C, D).



**Fig. 4.** Modifications of pH and lactic acid levels of *L. gasseri* CRL 1263, *L. reuteri* CRL 1324, *L. salivarius* CRL 1328 or *L. gasseri* CRL 1509 and *S. agalactiae* NH 17 in pure and mixed cultures. *Lactobacillus* strains in pure cultures with low inoculum (◆) or high inoculum (▲). Mixed cultures inoculated with low inoculum (●) or high inoculum (■) of lactobacilli. *S. agalactiae* NH 17 in pure culture (▶).



**Fig. 5.** (A, B) Viable cells of *S. agalactiae* NH 17 (NH 17) from murine vaginal washings of *Lactobacillus*–pathogen (Lb–Pt) experimental groups and pathogen (Pt) experimental group. Viable cells of *L. salivarius* CRL 1328 (CRL 1328) (C) and *L. gasseri* CRL 1509 (CRL 1509) (D) from murine vaginal washings of *Lactobacillus* (Lb) experimental group and Lb–Pt experimental groups. Mice in the Lb–Pt experimental groups were i.v. inoculated with CRL 1328 (A) or with CRL 1509 (B) and later challenged with NH 17. Mice in the Pt experimental group were challenged only with the pathogen. Mice in the Lb experimental groups were inoculated only with lactobacilli (CRL 1328 or CRL 1509). Data are plotted as individual (●) or mean values (○) of viable cell numbers of NH 17, CRL 1328 or CRL 1509. Statistically significant differences between the log (c.f.u. ml<sup>-1</sup>) of each experimental group on the different days post-inoculation are indicated by different letters ( $P < 0.05$ ). p.i., post-Lb inoculation; p.ch., post-Pt challenge.

The vaginal cytology and histology of mice confirmed their oestrous state, which was characterized by the presence of predominantly large, cornified cells. Also, a leukocyte influx was evidenced in Lb–Pt-treated mice and Pt-treated mice at day 1 post-pathogen challenge, but not on subsequent days. No such influx was observed in Lb-treated mice on any of the different sampling days (Figs S1 and S2).

## DISCUSSION

*Lactobacillus* strains can be selected as potential active ingredients for pharmabiotic products by means of *in vitro* and *in vivo* experimental animal assays in order to evaluate the beneficial characteristics believed to be involved in the protective mechanisms of the urogenital tract.

Some of the *in vitro* techniques relate to the interaction between beneficial strains and pathogens, including co-aggregation and production of antagonistic metabolites. Co-aggregation is a process in which genetically different micro-organisms become attached to one another via specific molecules (Younes *et al.*, 2012). In this work, the co-aggregation ability of lactobacilli with urogenital pathogens was evaluated. Out of the five *Lactobacillus* strains evaluated, *L. gasseri* CRL 1263, *L. reuteri* CRL 1324 and *L. gasseri* CRL 1509 were able to co-aggregate with at least two *S. agalactiae* strains. In contrast, Boris *et al.* (1998) showed that vaginal *Lactobacillus acidophilus*, *L. gasseri* and *Lactobacillus jensenii* were not able to co-aggregate with *S. agalactiae*.

Only *L. reuteri* CRL 1324 was capable of co-aggregating with *S. aureus*. In a similar way, experiments performed



with atomic force microscopy evidenced stronger adhesion forces between vaginal lactobacilli (mainly probiotic *L. reuteri* RC-14) and three virulent toxic shock syndrome toxin 1-producing *S. aureus* strains than between staphylococcal pairs, which result in larger lactobacilli–*S. aureus* co-aggregates (Younes *et al.*, 2012). None of the strains evaluated in this work co-aggregated with *C. albicans* strains. In contrast, Boris *et al.* (1998) showed that vaginal *L. acidophilus*, *L. gasseri* and *L. jensenii* were able to co-aggregate with *C. albicans*. Chassot *et al.* (2010) demonstrated that *C. albicans*–*L. acidophilus* co-aggregation caused a significant increase in the *in vitro* adhesion of the yeast and a decrease in the *Lactobacillus* adhesion to a contraceptive vaginal ring, suggesting that *L. acidophilus*-based probiotics could not exert a protective effect against *C. albicans*.

The results obtained in this work indicate that this phenomenon is strain specific and dependent on the time of the aggregation assay, as reported by other scientists (Collado *et al.* 2007; Ekmekci *et al.*, 2009). It is important to highlight that some UGTI, such as bacterial vaginosis, occur when co-aggregates are dominated by pathogens (MacPhee *et al.*, 2010). Moreover, when lactobacilli form co-aggregates with pathogens, homeostasis could be restored (Reid *et al.*, 1990). Younes *et al.* (2012) hypothesized that lactobacilli displaying strong adhesion forces with pathogens would facilitate co-aggregation and *in vivo* elimination of pathogens, partially explaining the efficacy of probiotic lactobacilli used to prevent UGTI.

With respect to antagonistic mechanisms, several *in vitro* studies have shown that vaginal *Lactobacillus* strains can inhibit the growth of a wide spectrum of urogenital pathogens, such as *S. agalactiae*, *S. aureus*, *Neisseria gonorrhoeae*, *E. coli* and *Klebsiella pneumoniae*, among others, through the production of different antimicrobial substances (organic acids, H<sub>2</sub>O<sub>2</sub> and bacteriocins) (Muench *et al.*, 2009; Bodaszewska *et al.*, 2010; Juárez Tomás *et al.*, 2011; Ruíz *et al.*, 2012). The results obtained in this work indicate that, out of the different urogenital pathogens assayed using the agar plate diffusion assay, only the *S. agalactiae* strains were inhibited by two vaginal *Lactobacillus* strains (*L. gasseri* CRL 1509 and *L. reuteri* CRL 1324) through the production of organic acids. Similarly, applying agar plate techniques, Ruíz *et al.* (2012) demonstrated that indigenous vaginal lactobacilli negatively affected the *in vitro* growth of *S. agalactiae* and that inhibition was caused by bacteriocin-like inhibitory substances produced by the lactobacilli.

When employing a more sensitive method (lactobacilli–pathogen associative cultures) than the diffusion test to study the antagonistic effects of lactobacilli on *Streptococcus agalactiae* NH 17, two different inoculum concentrations of lactobacilli were assayed according to the number of lactobacilli isolated in the genital tract of healthy fertile women ( $6.59 \pm 2.02$  log c.f.u. per vaginal sample; Rönnqvist *et al.*, 2006). The inoculum concentrations were also supported by the concept that high doses of probiotic

lactobacilli ( $10^6$ – $10^9$  viable cells per day), either taken orally or applied vaginally, are required to restore the ecological balance of the urogenital tract and to produce the physiological effect on the host (ISAPP, 2013; Mastromarino *et al.*, 2013). The *S. agalactiae* concentration used was selected to evaluate the inhibitory ability of lactobacilli against the highest number of the pathogen (5.0 log c.f.u. per vaginal sample) reported in women with positive samples (Rönnqvist *et al.*, 2006).

In lactobacilli–pathogen associative cultures, the four lactobacilli assayed significantly antagonized pathogen growth. Similar results were published by Bodaszewska *et al.* (2010), demonstrating a strong inhibitory activity of *Lactobacillus* species against *S. agalactiae* strains after 2 h of incubation in mixed cultures. This inhibition was dependent on the *Lactobacillus* strains tested, but independent of *S. agalactiae* serotypes evaluated. In humans, organic acids and H<sub>2</sub>O<sub>2</sub> produced by vaginal lactobacilli are considered the main factors that contribute to a hostile environment for pathogens (Reid & Burton, 2002; Martín *et al.*, 2008). Therefore, the levels of these metabolites that could participate in *S. agalactiae* NH 17 inhibition during associative cultures were determined. *L. gasseri* CRL 1509 (the strain that produced the highest lactic acid levels) showed the highest antagonistic effect on *S. agalactiae*, while *L. reuteri* CRL 1324 (the highest H<sub>2</sub>O<sub>2</sub> producer) exerted the lowest antagonistic effect, suggesting that this *S. agalactiae* strain could be sensitive to organic acids and resistant to H<sub>2</sub>O<sub>2</sub>. Liu *et al.* (2004) demonstrated that *S. agalactiae* is capable of producing a carotenoid pigment with antioxidant properties that affords protection against reactive oxygen species (e.g. H<sub>2</sub>O<sub>2</sub>), important components of the oxidative burst killing mechanisms of host phagocytic cells, which could explain the results obtained in this work.

The *in vitro* studies indicate that *L. gasseri* CRL 1509 and CRL 1263, *L. salivarius* CRL 1328 and *L. reuteri* CRL 1324 produce antimicrobial substances active against *S. agalactiae*. Moreover, the significant co-aggregation between *Lactobacillus* (mainly CRL 1509, CRL 1263 and CRL 1324) and *S. agalactiae* strains could facilitate the inhibitory effect and the elimination of the co-aggregation partner from the vaginal tract. These results could be clinically relevant since *S. agalactiae* is an opportunistic pathogen that can cause vaginitis, painful fine superficial fissures and minimal erythema of vulvar skin in adolescent and young adult populations as well as serious invasive infections in susceptible adult populations. In pregnant women, the presence of *S. agalactiae* is associated with the risk of neonatal infections (bacteraemia, sepsis, pneumonia and/or meningitis) and death (Clark & Atendido, 2005; Maisey *et al.*, 2008; Rajagopal, 2009; Mirowski *et al.*, 2012; Savini *et al.*, 2013).

Therefore, pharmabiotic products containing beneficial *Lactobacillus* strains could be a promising alternative for protection against *S. agalactiae* in women, thus preventing

*S. agalactiae* infections in newborns. However, further testing on animals is required previous to clinical studies and inclusion of the strains in the design of such products.

Unlike the *in vitro* inhibition of the pathogens, where *L. gasseri* CRL 1509 and *L. salivarius* CRL 1328 showed co-aggregative and antimicrobial properties on *S. agalactiae* NH 17, these strains were not able to inhibit vaginal colonization of the pathogen in the experimental murine model employed. Several conditions could favour the *in vitro* inhibitory effect of *Lactobacillus* on the pathogen, but they would be different from the ones in the murine vaginal ecosystem of the model used in our work.

In a similar way, Muench *et al.* (2009) reported that *Lactobacillus crispatus*, a human vaginal H<sub>2</sub>O<sub>2</sub>-producing *Lactobacillus* strain, inhibited *N. gonorrhoeae in vitro* but showed no evidence of a difference either in the duration of vaginal infection or in the number of gonococci recovered from the vaginal tract of oestrogenized *Lactobacillus*-treated or untreated mice. Moreover, the i.v. inoculation of mice with 10<sup>7</sup> c.f.u. of human *Lactobacillus* strains (vaginal *L. jensenii* ATCC 25258 or urine *L. crispatus* ATCC 33197) did not produce a decrease in vaginal pH compared with the vaginal pH (around 6.0–6.8) of buffer-inoculated control mice (Muench *et al.*, 2009). Based on the fact that murine vaginal pH is higher than normal human vaginal pH (around 4.0–4.5) (Larsen, 1993) and taking into account that acidity favours the stability of inhibitory substances, Muench *et al.* (2009) hypothesized that lactobacilli could be less toxic in the less acidic environment of the murine vagina.

Even though several research works have demonstrated the *in vitro* effects of vaginal lactobacilli on *S. agalactiae* (Bodaszewska *et al.*, 2010; Juárez Tomás *et al.*; 2011; Ruiz *et al.*, 2012), the *in vivo* effect of lactobacilli on this genital pathogen has not been assessed up to now. Cheng *et al.* (2005) and Cavaco *et al.* (2013) used murine models of *S. agalactiae* vaginal colonization to evaluate the effect of a bacteriophage lytic enzyme and an immunobiotic peptide. Both studies evidenced a reduction in *S. agalactiae* vaginal colonization. However, this is to our knowledge the first work evaluating the *in vivo* effect of vaginal lactobacilli on *S. agalactiae* colonization, although no positive result could be achieved.

In conclusion, different results were obtained through *in vitro* and *in vivo* assays: human vaginal lactobacilli exhibited *in vitro* antimicrobial effects on *S. agalactiae*, but these effects were not evidenced in the murine model used. Thus, further animal studies under different experimental conditions should be carried out. Inoculation of mice with higher doses of lactobacilli could be assayed in order to determine if higher inocula or viable cell numbers could favour the colonization/permanence of lactobacilli and the production of antimicrobial substances (e.g. organic acids acidifying the murine vaginal milieu) that can afford protection against pathogens. Also, some experiments could be designed with previous modification

of the pH of the vaginal media, or suspending the micro-organisms in a low pH medium, to determine if these conditions would favour persistence of lactobacilli and their protective effect.

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